American Chemistry Council
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September 9, 2005

VIA HAND DELIVERY

TSCA Confidential Business Information Center (7407M)
EPA East - Room 6428 Attn: Section 8(e)
1201 Constitution Ave NW
Washington DC 20004-3302
(202) 564-8940

Attention: TSCA 8(e) Coordinator

RE: Information Acquired During Study with Diphenylmethane-4,4'-Diisocyanate (Polymeric MDI)

Dear TSCA 8(e) Coordinator:

The American Chemistry Council's Diisocyanates Panel (Panel) is submitting on behalf of its members[1] the results of a study with diphenylmethane-4,4'-diisocyanate (polymeric MDI)[2] (CAS No. 9016-87-9). The objective of this study, titled "Lung Sensitization Study in Brown-Norway Rats Following Either Topical Induction and Repeated Inhalation Challenges" (*see* attachment), was to evaluate the asthmagenic potential of MDI using Brown Norway rats sensitized to MDI, administered by topical exposures. The data provided herein are being submitted pursuant to Section 8(e) of the Toxic Substances Control Act (TSCA).

The study author summarizes the results of the study as follows:

> During the sensitization phase some animals displayed dose-dependent local effects at the site of induction. After challenge transient breathing responses were observed. In topically sensitized rats, a time-related exacerbation of delayed-onset responses were observed. Pulmonary inflammation was indicated by most endpoints determined in bronchoalveolar lavage. This included elevated protein, increased numbers of neutrophilic and eosinophilic granulocytes and lymphocytes.

[1] The members of the Panel are BASF Corporation, Bayer MaterialScience, Dow Chemical Company, Huntsman Polyurethanes, and Lyondell Chemical Company.

[2] J. Pauluhn, Polymeric MDI: lung sensitization study in Brown-Norway rats following topical induction and repeated inhalation challenge. III Report 11511, August 2005, International Isocyanate Institute Inc., Manchester, UK.

Histological inflammatory findings (focal inflammatory infiltrates, inflammation) were seen especially in sensitized rats. These lesions included inflammation of the airways, partly with beginning peribronchial/peribronchiolar fibrosis and included alveolar septal thickening and BALT activation. IgE determinations revealed slightly elevated levels in the topical sensitization groups. In the topical-high group the increase gained statistical significance.

In summary, the findings of this study support the conclusion that the Brown Norway rat model is suitable to identify MDI as an agent causing a pulmonary inflammatory response upon topical induction followed by repeated inhalation challenge exposures. Consistent and unequivocally positive delayed-type changes of breathing patterns were observed. These findings suggest that MDI promotes a more delayed-onset type rather than immediate-type inflammatory response.

While being submitted in accordance with TSCA 8(e), the Panel has made no determination as to whether a substantial risk of injury to health or the environment is actually presented by these findings.

If you have any questions, please contact me, the Diisocyanates Panel Manager, at 703-741-5635 or susan_lewis@americanchemistry.com.

Best regards,

Susan A. Lewis

Susan Anderson Lewis, Ph.D.
Manager, Diisocyanates Panel

Attachment
Cc: DII Panel

III Project 247

III Report 11511

Polymeric MDI: lung sensitization study in Brown-Norway rats following topical induction and repeated inhalation challenge

J Pauluhn

Bayer HealthCare
Wuppertal
Germany

Issued: August 2005

Number of pages: 194

III Report

International Isocyanate Institute Inc.

The Scientific Office, Bridgewater House, Whitworth Street, Manchester M1 6LT. UK.

The Scientific Office of the International Isocyanate Institute Inc.
is operated by Gilbert International Limited, an independent contractor.

This report is on research sponsored by the International Isocyanate Institute, Inc.

The information, analysis, methods and recommendations herein are presented in good faith, are believed to be accurate and reliable, but may well be incomplete and/or not applicable to all conditions or situations that may be encountered.

No representation, guarantee or warranty is made as to the accuracy, reliability or completeness of this report, or that the application or use of any of the information, analysis, methods and recommendations herein will avoid, reduce or ameliorate hazards, accidents, losses, damages or injury of any kind to persons or property. Readers are therefore cautioned to satisfy themselves as to the applicability and suitability of said information, analysis, methods and recommendations for the purposes intended prior to use.

BAYER HEALTHCARE AG
PH-PD-TOXICOLOGY INTERNATIONAL
D - 42096 WUPPERTAL

Report-No.:

DIPHENYLMETHANE-4,4'-DIISOCYANATE
(polymeric MDI)

Lung Sensitization Study in Brown-Norway Rats following either Topical Induction and repeated Inhalation Challenges

STUDY DIRECTOR

Prof. Jürgen Pauluhn Ph.D., D.A.B.T.

SPONSOR

INTERNATIONAL ISOCYANATE INSTITUTE
2805 East Dupont Road
Ft. Wayne, IN 46845
U.S.A.

Bayer Project-no.: T1074866 / III-Proj. 247 EU-MTX

Study Completion Date:July 04, 2005

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GLP COMPLIANCE STATEMENT

This study was not conducted in compliance with the OECD Principles of Good Laboratory Practice as revised in 1997 (ENV/MC/CHEM(98)17) and with the revised German Principles of Good Laboratory Practice according to Annex I German Chemicals Act (Bundesgesetzblatt Part I, No. 40 issued June 27, 2002) and served the purpose of a principle validation of the techniques and regimens used for induction and the identification of delayed-onset physiological responses.

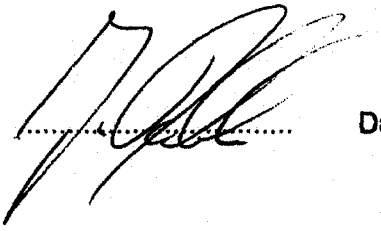
Prof. Dr. J. Radühn D.A.B.T.
Board Approved Toxicologist (DGPT)
EUROTOX Registered Toxicologist
Study Director

Date: May 12, 2005

1. TABLE OF CONTENTS

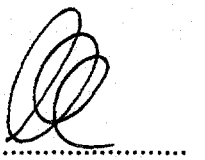
2. SIGNATURES

Study Director - Prof.Dr. J. Pauluhn D.A.B.T.:



Date: July 4, 2005

Head of Section - Dr. Dr. H.-J. Ahr:



Date: May 12, 2005

3. SUMMARY

Brown Norway rats (eight male rats per group) were sensitized topically as follows: day 0: 40 or 150 µl of undiluted polymeric methylenediphenylene diisocyanate (abbreviated MDI) was administered topically on the flanks, day 7: booster administration to the skin using the same dose on the contralateral flank. Throughout the report the 40 and 150 µl/animal groups are abbreviated as topical-low and topical-high, respectively. The control-1 group was not sensitized nor challenged, whilst the control-2 group was challenged similarly as the topical groups. On days 16, 35, 49, 65 (±3 days) the rats were challenged (except control-1) with MDI aerosol (38 mg/m^3, duration: 30-min). After the MDI challenge basic respiratory function parameters were measured for approximately 20 hours before and after challenge at the last challenge in the control-2 and at all challenges in the topical-high group. After the last MDI-challenge, the rats were sacrificed, lung weights were determined. Lungs were lavaged for the analysis of inflammatory endpoints. Inflammatory endpoints were determined in bronchoalveolar lavage fluid (BAL). Total IgE was analyzed in serum. Histopathology of the lung was made on tissues collected (all rats).

The results of study can be summarized as follows: During the sensitization phase some animals displayed dose-dependent local effects at the site of induction. After challenge transient breathing responses were observed. In topically sensitized rats, a time-related exacerbation of delayed-onset responses were observed. Pulmonary inflammation was indicated by most endpoints determined in bronchoalveolar lavage. This included elevated protein, increased numbers of neutrophilic and eosinophilic granulocytes and lymphocytes. Histological inflammatory findings (focal inflammatory infiltrates, inflammation) were seen especially in sensitized rats. These lesions included inflammation of the airways, partly with beginning peribronchial/peri-bronchiolar fibrosis and included alveolar septal thickening and BALT activation.

IgE determinations revealed slightly elevated levels in the topical sensitization groups. In the topical-high group the increase gained statistical significance.

In summary, the findings of this study support the conclusion that the Brown Norway rat model is suitable to identify MDI as an agent causing a pulmonary inflammatory response upon topical induction followed by repeated inhalation challenge exposures. Consistent and unequivocally positive delayed-type changes of breathing patterns were observed. These findings suggest that MDI promotes a more delayed-onset type rather than immediate-type inflammatory response.

4. INTRODUCTION

The objective of this study was to evaluate the asthmagenic potential of MDI using Brown Norway rats sensitized to MDI, administered by topical exposures. For the elicitation of respiratory allergy a more chronic repeated challenge protocol was chosen. The advantage of a repeated challenge protocol is that features characteristic of the allergic airway, that include airway remodeling and sustained recruitment of inflammatory cells, can be evaluated and assessed.

Testing facility:

The study was conducted at Bayer HealthCare AG, PH-PD Toxicology International / Inhalation Toxicology, D-42096 Wuppertal, Germany.

Study/project identification:

Bayer Project-no.:	T1074866
III- Project-no.	247 EU-MTX
Experimental starting date:	September 28, 2004
Experimental completion date:	March 22, 2005 (Histopathology Report)
Study completion date:	see signature of study director (page 7)

5. RESPONSIBILITIES

Air conditioning/air make-up ..... D.I. F.-W. Mentzel
Archiving of raw data and report: ..... R. Zils
Analytical characterization of test article: ..... Dr. Müller/BPO-GO-MDI-PT-UER-QSU
Biometric Evaluation of Data: ..... Prof. Dr. Dr. J. Pauluhn
Bronchoalveolar lavage: ..... Drs. I.Loof
Cell count & cytospins: ..... Dr. G.Wasinska-Kempka
Gross Pathology & Histopathology: ..... Dr. M. Rosenbruch
Head of Institute: ..... Dr. v.Keutz
Head of Section: ..... Dr.Dr. H-J.Ahr
Immunological Determinations: ..... Prof. Dr. H.W.Vohr
Laboratory Animal Services: ..... Dr. W. Feller
Quality Assurance: ..... Dr. H. Lehn
Study Monitor: ..... Dr. Mike Woolhiser, Dow Chemicals, U.S.A.
Study Director and Report Author: ..... Prof. Dr. J. Pauluhn

6. MATERIALS AND METHODS

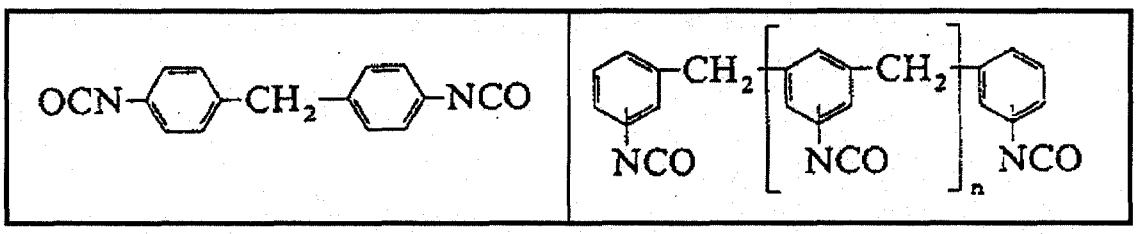
6.1. Test Substance

Chemical name:	Diphenylmethane-4,4'-diisocyanate (MDI-polymer)
Abbreviation:	MDI
Commercial name:	DESMODUR® V 20 L
Batch-no.:	P4DB000244 (Tox Id: 8841)
Purity:	44% Monomer (see pp. 192)
Date of production:	July 07, 2005, shelf life: up to January 3, 2005.
Manufacturer:	BAYER Polymers AG, Leverkusen, Germany
Storage conditions:	refrigerator (≈ 4 °C) / darkness / N_2-atmosphere {prior to study}
Storage conditions:	at room temperature {during study}.
Handling:	complete exclusion of air/humidity (handling and storage in dry nitrogen)
Appearance:	brownish, translucent liquid material (viscous)

Molecular formula (*of monomer*): $C_{15}H_{10}N_2O_2$

Molecular weight: 250.3 g/mol (monomer)

Representation of *monomeric* (left panel) and *polymeric* MDI (right panel) in their generic forms:



6.2. Test system and animal maintenance

Species: Male Brown Norway rats of the strain BN/Crl BR were purchased from Charles River, Sulzfeld, Germany. At the commencement of the study body weights were approximately ≈230 g (± 5%).

Acclimatization: The animals were acclimatized to the animal room conditions for approximately 1 week before.

Identification: Animals were identified by both individual color-marking and cage-labels.

Randomization: Before the start of the study the health status of each animal was assessed. Animals were subsequently assigned to exposure groups at random (randomization procedure *vide infra*).

Health status: Only healthy animals free of signs were used for this study. The animals were not vaccinated or treated with anti-infective agents either before their arrival or during the acclimatization or study periods.

Animal housing: During the acclimatization and study periods the animals were housed singly in conventional Makrolon® Type II cages (based on A. Spiegel and R. Gönnert, Zschr. Versuchstierkunde, 1, 38 (1961) and G. Meister, Zschr. Versuchstierkunde, 7, 144-153 (1965)). Cages were changed twice a week while unconsumed feed and water bottles were changed once per week. The legal requirements for housing experimental animals (Directive 86/609 EEC) were followed.

Bedding: Bedding consisted of type BK 8/15 low-dust wood granulate from Ssniff, Soest/Westfalen, Germany. The wood granulate was randomly checked for harmful constituents at the request of the Laboratory Animal Services, Bayer HealthCare AG.

Animal rooms: All animals were housed in a single room.

Environmental Conditions in the Animal Room

The animal room environment was as follows:

Room temperature:	22 ± 2 °C
Relative humidity:	approximately 50 %
Dark/light cycle:	12 h/12 h; artificial light from 6.00 a.m. to 6.00 p.m. Central European Time
Light intensity:	approximately 14 watt/m² floor area
Ventilation:	approximately 10 air changes per hour

The room humidity and temperature were continuously monitored and documented using a calibrated thermohygrograph. Occasional deviations from these conditions

occurred, e.g. as a result of animal room cleaning, but these had no detectable influence on the outcome of this study.

Cleaning, disinfection, and pest control: The animal room was regularly cleaned and disinfected once a week with neat TEGO® 2000. Contamination of the feed and contact with the test system were excluded. Pest control measures using pesticides were not taken in the animal room.

Feeding: Ration consisted of a standard fixed-formula diet (KLIBA 3883 = NAFAG 9441 pellets maintenance diet for rats and mice; PROVIMI KLIBA SA, 4303 Kaiseraugst, Switzerland) and tap water (drinking bottles). Both food and water were available *ad libitum*. The pelletized feed was contained in a rack in the stainless-steel wire cage cover. The nutritive composition and contaminant content of the standard diet was checked regularly by random sampling by the Laboratory Animal Services, Bayer HealthCare AG. Details concerning general feed specification are provided in the Appendix.

Water: Drinking quality municipality tap-water (current versions of the Drinking Water Decree (TrinkwV)) was provided *ad libitum* in polycarbonate bottles containing approximately 300 ml (based on A. Spiegel and R. Gönnert, Zschr. Versuchstierkunde, 1, 38 (1961) and G. Meister, Zschr. Versuchstierkunde, 7, 144-153 (1965)). The results of feed and water analyses are retained by Bayer HealthCare AG. The available data provided no evidence of an impact on the study objective.

6.3. Exposure Regimen, Dose Selection, Study Rationale

1) **Induction:** Two topical treatment groups of either 40 and 150 µl MDI/rat (undiluted test article) was applied to the flank followed by a booster induction on day 7 on the contralateral flank using the same dose. Two additional groups served as sham control groups (control-1: naive rats that were not re-challenged (C-/-), whilst the control-2 rats were re-challenged at all time points (C-/+)).

2) **Elicitation of changes in breathing patterns:** The respective rats were challenged by inhalation on the targeted days (±4) 16, 35, 49, and 69 with approximately 40 mg MDI/m³ for 30-min each. After the last challenge the rats were sacrificed for specialized examinations (see below).

3) **Endpoints (after the last challenge):**

- Challenge with MDI followed measurements of respiratory rate, tidal volume, and Penh for approximately 20 hours after challenge (for details see Appendix/Calendar). Measurements were also made the days before challenge. As no differences were observed the day shortest to the MDI-challenge was selected and reported. Due to technical reasons these measurements were made in the topical high (all challenges) and C-/+ (last challenge only) groups.
- Approximately two days after the MDI-challenge: Lung lavage and determination of inflammatory endpoints in bronchoalveolar lavage fluid (BALF): lactate dehydrogenase (LDH), and protein. Total cell counts and cytodifferentiation of cytospins.

- Total serum IgE (all groups) after the last challenge.
- Lungs, after complete exsanguination of animals, were weighed. Neutral, phosphate-buffered 10% (v/v) formaldehyde was used to inflate and to preserve the lungs, including trachea. These organs were examined in all groups on animals sacrificed after the first and last challenge.

4) **Protocol (see also Figs. 1 and 2):**

Assignment of animals:

1: Sham control-1 (C-/-): rat nos. : 1 - 8
2: Sham control-2 (C-/+): rat nos. : 9 - 16
3: Topical-low: rat nos. :17 - 24
4: Topical-high:: rat nos. : 25 - 32

Figure 1: Principle test protocol

Principle of Animal Model – 231 vs. 247 EU-MTX

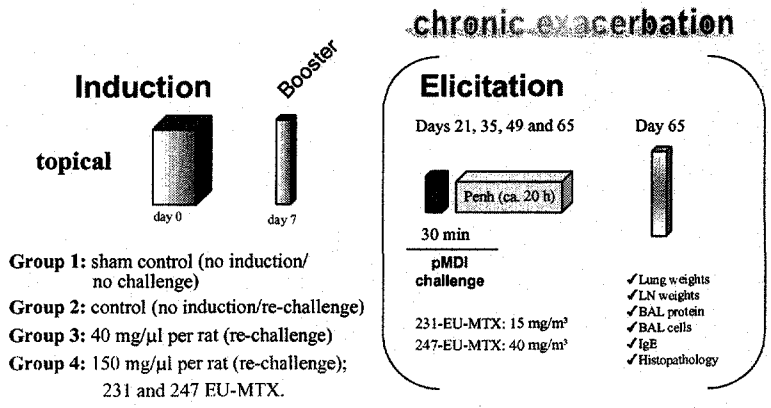
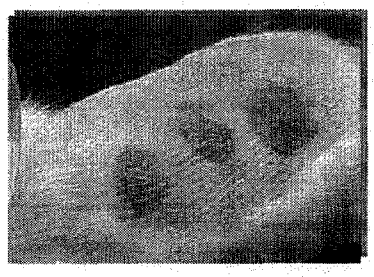


Figure 2: Principle induction regimen used

Principle of Animal Models – 231 vs. 247 EU-MTX

■ **231-EU-MTX:**
- Induction: flanks (150 µl/animal & 5-10 cm²)
- Booster: dorsum of ears (2 x 75 µl/ear)

■ **247-EU-MTX:**
- Induction: flank-left (40 or 150 µl/animal)
- Booster: flank-right (40 or 150 µl/animal)
  - 40 µl: 4 x 10 µl/1 cm spot
  - 150 µl: 3 x 50 µl/2 cm spot



6.4. Study design

The protocols utilized to sensitize Brown Norway rats to MDI were largely consistent with the methods described previously for similar investigations in Brown Norway rats sensitized to trimellitic anhydride (TMA) (Pauluhn et al., 2002).

This study consisted of two naïve control groups and two groups of BN rats that were sensitized epicutaneously on days 0 and 7. Each group consisted if eight male rats allocated to the four groups by randomization. For topical sensitization a slightly modified methodology from a previous study with MDI was used in order to improve the control over the administered dose per surface area (SA). On days 0, the rats of the topical-low and topical-high groups received either 40 or 150 µl MDI on the left-dorsal area of the trunk. The same dose was administered to the contralateral flank on day 7 as booster, instead of applying half of the dose of day 0 on the dorsum of each of both ears (as used in the previous study 231-EU-MTX, see Fig. 2). The different doses were administered by using aluminum foil spots of either 1 or 2 cm in diameter. After metering a predefined volume of MDI to each foil the weight of MDI was determined using a digital balance. Then the test substance was transferred to the skin by pressing the spot onto the skins' surface and was then removed. In the topical-low and topical-high groups per application site 4 x 1 cm spots, each with 10 µl of MDI (cumulative dose: 127.6±10.6 mg/rat; cumulative dose/SA: 20.3±1.7 mg/cm^2) or 3 x 2 cm spots each with 50 µl of MDI (cumulative dose: 398±7.0 mg/rat; cumulative dose/SA: 21.1±0.4 mg/cm^2) were used, respectively. The skin was shaved 1 day prior to administration. Rats were prevented from grooming or scratching by wearing an Elizabean collar up to the morning the day following administration (Buster Birdcollars; Kruuse, DK, Cat no.: 273375).

The C-/- control group was neither sensitized nor challenged at any time point, whilst C-/+ control, the topical-low and topical-high were repeatedly challenged with MDI aerosol on days 16, 35, 49, and 63 ((C-/+) or 69 (topical induction groups) for 30-min. These groups were challenged simultaneously in one single nose-only inhalation chamber (except on day 63/69). Four out of the eight rats of the topical-high group were monitored for 20 hours after each challenge one day before and shortly after the MDI challenge for delayed onset respiratory effects. Animals of the C-/+ control group were challenged in the same way, however, measurements for delayed-onset responses were made only the day before and shortly after the MDI challenge made on day 63. Two days after the last challenge, rats were sacrificed, the weights of exsanguinated lungs were determined and the lungs were lavaged for the analysis of endpoints suggestive of an inflammatory response. Lavaged lungs were examined by conventional histopathology. At sacrifice blood was collected by heart puncture for total IgE determination in serum.

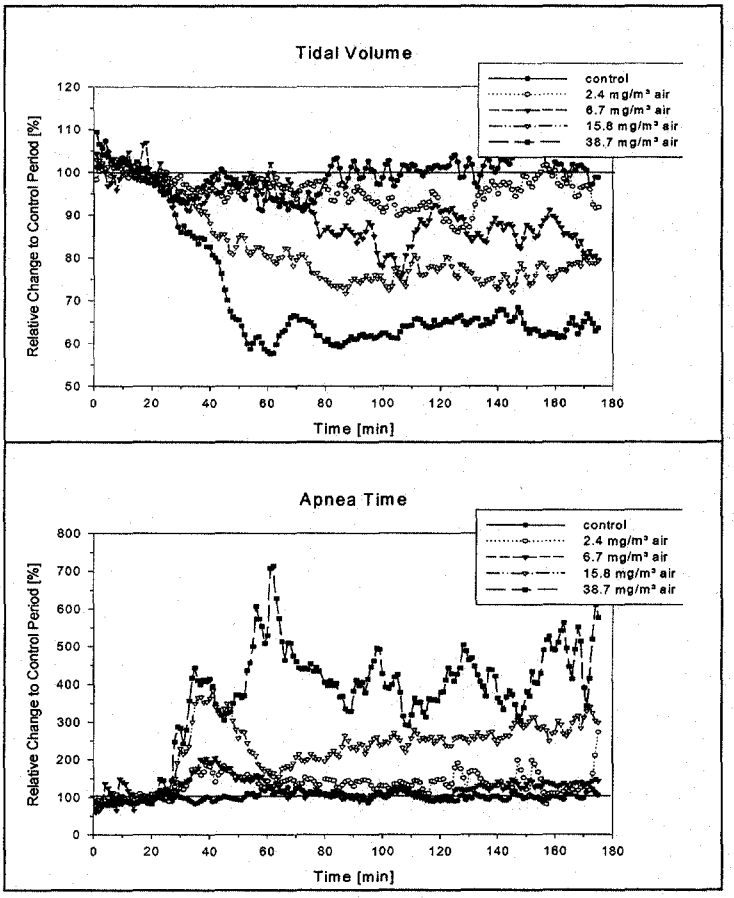
Table 1: Topically administered volumes/doses of MDI.

Animal no.	Area/ Spot cm^2	Spots/ diameter #/cm	Vol./ Spot µl	Mass/ Spot mg	Mass/ Rat mg	Cum Mass/ mg	Cum Mass/ mg/cm^2
				Day 0	Day 7	Rat	SA
17	0.785	4/1	10	60.30	55.80	116.10	18.49
18	0.785	4/1	10	65.70	52.20	117.90	18.77
19	0.785	4/1	10	67.30	51.30	118.60	18.89
20	0.785	4/1	10	71.40	67.00	138.40	22.04
21	0.785	4/1	10	67.20	60.60	127.80	20.35
22	0.785	4/1	10	70.80	65.10	135.90	21.64
23	0.785	4/1	10	57.70	64.50	122.20	19.46
24	0.785	4/1	10	75.20	68.80	144.00	22.93
Mean				**66.95**	**60.66**	**127.61**	**20.32**
SD				**5.78**	**6.80**	**10.63**	**1.69**
25	3.14	3/2	50	210.50	185.20	395.70	21.00
26	3.14	3/2	50	204.90	192.50	397.40	21.09
27	3.14	3/2	50	210.90	200.40	411.30	21.83
28	3.14	3/2	50	209.60	193.80	403.40	21.41
29	3.14	3/2	50	214.50	178.80	393.30	20.88
30	3.14	3/2	50	211.00	189.30	400.30	21.25
31	3.14	3/2	50	202.40	192.30	394.70	20.95
32	3.14	3/2	50	204.40	184.20	388.60	20.63
Mean				**208.53**	**189.56**	**398.09**	**21.13**
SD				**4.14**	**6.71**	**6.95**	**0.37**

Repeated challenge exposures were with approximately 40 mg/m^3 which is an irritant concentration (Fig. 3).

With regard to irritant-related acute changes in breathing patterns, Wistar rats exposed for approximately 150 min to a concentration of ≈16 mg MDI/m^3 elaborated marginal effects, whilst the exposure to 39 mg MDI/m^3 caused distinct changes in breathing patterns suggestive of lower respiratory tract irritation (Pauluhn, 2000). In contrast to the previous sensitization study in Brown Norway rats with MDI (Pauluhn et al., 2005) in the current study the challenge concentration was increased from 16 to 39 mg/m^3 under otherwise identical inhalation exposure conditions. Details of the topical test regimen are shown in Table 1.

Figure 3: Analysis of the dependence of respiratory tidal volume and apnea time on various concentrations of MDI-aerosol. The data shown represent the means of six Wistar rats per group exposed in nose-only volume displacement plethysmographs for approximately 140-min. Prior to MDI exposure the rats were acclimatized for approximately 35-min while exposed to air (no data shown). Data were averaged for time periods of 1-min. Body weights were approximately 215 gram (mean ± SD). Data from study no.: T5062502.



6.5. Aerosol Generation and Exposure Technique

Mode of exposure: Animals were exposed to the aerosolized test substance in restrainers made of Plexiglas. Restrainer tubes were chosen that accommodated the animal's size. The design of the *directed-flow* inhalation chamber prevents re-breathing of the test atmosphere (Moss and Asgharian, 1994). This type of exposure is preferable to whole-body exposure on scientific (Pauluhn, 1984) and technical reasons (rapid attainment of steady-state concentrations, no problems with regard to test atmosphere inhomogeneities, better capabilities to control all inhalation chamber

parameters, easier cleaning of exhaust air, and lower consumption of test item). Moreover, contamination of the hair-coat can largely be avoided. The operation of this commercially available chamber (TSE company in Bad Homburg v.d.H., Germany) and its validation has been published in detail (Pauluhn, 1994).

Generation of atmosphere: Atmospheres of MDI for inhalation exposures were generated under dynamic conditions using a digitally controlled Hamilton Microlab M pump and a modified Schlick-nozzle Type 970, form-S 3 (Schlick GmbH, Coburg, Germany).

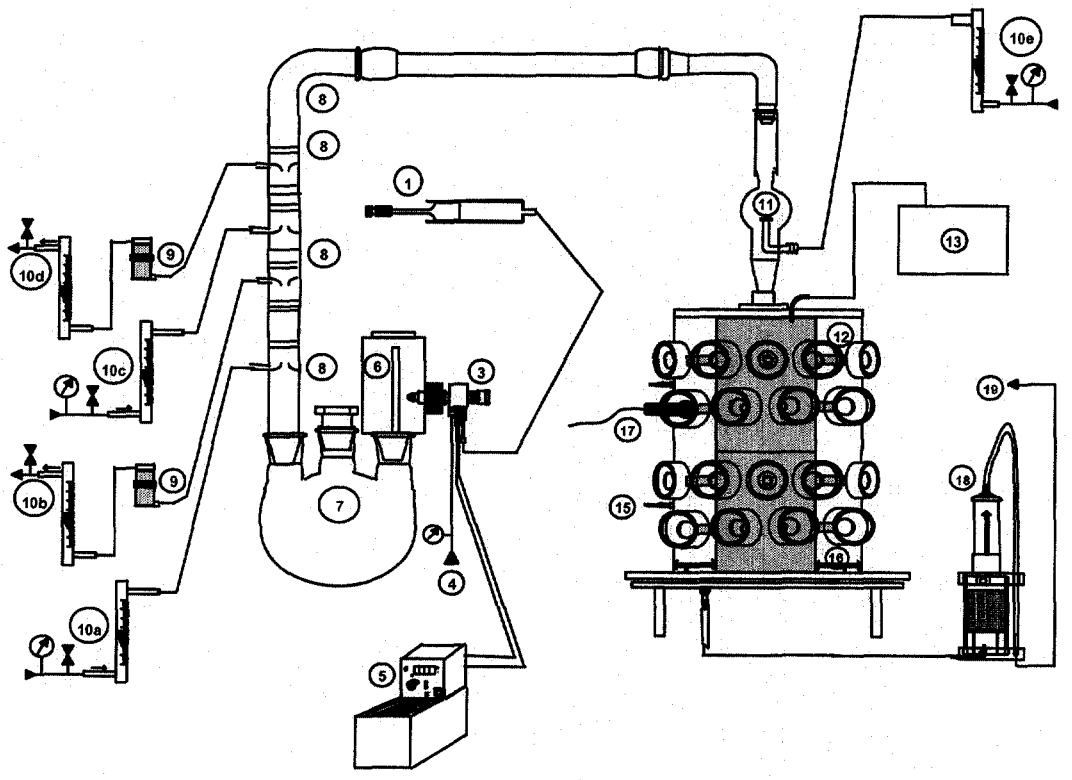
Generation of aerosol: The test substance was nebulized using conditioned (dry, oil-free) compressed air (dispersion pressure approximately 600 kPa, 10 µl MDI/min, 15 L/min and inhalation chamber segment). The nozzle were maintained at approximately 40 °C by a water jacket connected to a digitally controlled JULABO thermostat. The increase of temperature within the nozzle resulted in a marked decrease in viscosity and hence increased reproducibly the output of aerosol. The respective concentration was achieved by applying the extraction/dilution cascades depicted in Fig. 4.

Inhalation Chamber: Each segment of the aluminum inhalation chamber has the following dimensions: inner diameter = 14 cm, outer diameter = 35 cm (two-chamber system), height = 25 cm (internal volume = about 3.8 L). The construction of the inhalation chamber is shown schematically in Fig. 5. For this study a two segment-chamber was used. Flow rates through the inhalation chamber were 30 L/min. Further details are presented in Table 1.

Compressed air conditioning: The compressed air was produced with two Boge Model SB 270/15/350D compressors operated in parallel. The air was automatically conditioned (i.e. water, dust and oil removed) by subsequent passage through a VIA compressed air dryer. The regulated operating pressure of the compressors was 8 - 10 bars (800 - 1000 kPa). Pressure-reduction valves were used to set the operating pressure.

Inhalation chamber - steady-state concentration: The test atmosphere generation conditions assured at least 230 air volume exchanges per hour. A steady state was established in less than approximately one minute of operation under these test conditions ($t_{95\%}$ = 3 x chamber volume/air flow rate; McFarland, 1976). The ratio of input to exhaust air was selected to ensure that approximately 90% of the input air was removed by the exhaust system, and the remainder via other chamber openings. An air flow towards the rats' exposure zones was thus provided in the exposure system (*directed-flow* principle) allowing an adequate ventilation of the animals' breathing zone.

Figure 4: Inhalation Chamber (schematic)



1. MDI-reservoir and Harvard PHD 2000 Pump	11. Mixing unit (glass reservoir)
3. Schlick-nozzle (@ 40°C)	12. Directed-flow nose-only exposure zone
4. Pressurized, dry, conditioned air with pressure gauge	13. Photometer (real-time aerosol monitoring)
5. JULABO thermostat– water bath (water jacket)	15. Sampling for nitro-reagent/filter analyses
6. PVC – pre-separator	16. Inhalation chamber exhaust location
7. Mixing unit (3-neck glass flask)	17. Temperature-/humidity sensor
8. Dilution cascade	18. Cotton-wool aerosol filter + HEPA filter
9. Cotton-wool aerosol filter	19. Exhaust air
10.a-e. Dilution air flows	

Air flows: During the exposure period air flows were monitored continuously and, if necessary, readjusted to the conditions required. Air flows were measured with calibrated flow-meters and/or soap bubble meter (Gilibrator, Ströhlein Instruments, Kaarst) and were checked for correct performance at regular intervals.

Treatment of exhaust air: The exhaust air was purified via cotton-wool/HEPA filters. These filters were disposed of by Bayer AG.

6.6. Inhalation Chamber Temperature and Humidity

Temperature and humidity measurements were made using a computerized system (Hydra, Fluke-Philips). The values were recorded at intervals of 5 min (computerized recording). The test atmosphere temperature and humidity were measured at the exposure location (see Fig. 4). Humidity and temperature were measured using a FTF-sensor (Elka-Elektronik, Lüdenscheid). The sensor was calibrated using saturated salt solutions according to Greenspan (1977) and Pauluhn (1994) in a two-point calibration at 33% ($MgCl_2$) and at 75% (NaCl) relative humidity. The calibration of the temperature sensor is also checked at two temperatures using reference thermometer. The measured values were evaluated using spreadsheet software.

6.7. Analysis of the Test Atmosphere

Nominal concentration: The nominal concentration was calculated from the ratio of the quantity of test item atomized. Specific information concerning air flows and test atmosphere concentrations are provided in Table 3.

Gravimetric concentration: The test-item concentration was determined by gravimetric analysis (filter: Glass-Fibre-Filter, Sartorius, Göttingen, Germany; digital balance). The total volume sampled per analysis was 50 L (sampling flow rate 4 L/min).

Chamber samples were taken in the vicinity of the breathing zone (see Fig. 4). The number of samples taken was sufficient to characterize the test atmosphere and was adjusted so as to accommodate the sampling duration and/or the need to confirm specific concentration values. Optimally, samples were collected after the equilibrium concentration had been attained in hourly intervals. All analytical concentrations reported refer to mg MDI/m^3 air.

6.8. Characterization of Aerodynamic Particle-Size Distribution

The samples for the analysis of the particle-size distribution were also taken in the

vicinity of the breathing zone. During each exposure two samples were taken.

The particle-size distribution was analyzed using a BERNER-TYPE AERAS low-pressure critical orifice cascade impactor (Hauke, Gmunden, Austria). Specifications and evaluations are provided in the Appendix. The individual impactor stages had been covered by an aluminum foil which was subjected to gravimetric analysis An adhesive stage coating (silicone spray) was not used to prevent particle bounce and re-entrainment because of the physical properties of the test compound. Gravimetric analyses were made using a digital balance.

The parameters characterizing the particle-size distribution were calculated according to the following procedure:

Mass Median Aerodynamic Diameter (MMAD): Construct a 'Cumulative Percent Found - Less Than Stated Particle Size' table, calculate the total mass of test item collected in the cascade impactor. Start with the test item collected on the stage that captures the smallest particle-size fraction, and divide this mass of the test item by the total mass found above. Multiply this quotient by 100 to convert to percent. Enter this percent opposite the effective cut-off diameter of the stage above it in the impactor stack. Repeat this step for each of the remaining stages in ascending order. For each stage, add the percentage of mass found to the percentage of mass of the stages below it. Plot the percentage of mass less than the stated size versus particle size in a probability scale against a log particle-size scale, and draw a straight line best fitting the plotted points. A weighted least square regression analysis may be used to achieve the best fit. Note the particle size at which the line crosses the 50% mark. This is the estimated Mass Median Aerodynamic Diameter (MMAD).

Calculation of **Geometric Standard Deviation (GSD)**: Refer to the log probability graph used to calculate the Mass Median Aerodynamic Diameter. Provided that the line is a good fit to the data, the size distribution is log normal, and the calculation of the Geometric Standard Deviation is appropriate. Note that particle size at which the line crosses the 84.1% mark. Note the particle size at which the line crosses the 50% mark and calculate as follows: GSD = 84.1% mark / 50% mark.

To verify graphically that the aerosol is in fact unimodal and log-normally distributed the normalized mass per stage (f_H') is evaluated as a histogram. $\Delta \log D_p$ is equal the difference $\log D_{p+1} - \log D_p$, whereas D_p is the lower cut-size limit and D_{p+1} the higher cut-size limit of the corresponding impactor stage. Calculate the histogram f_H' by equation:

$$f_H' = \frac{1}{N_f} \times \frac{mass \ / \ stage}{\Delta \log D_p} \qquad (1)$$

Calculate the log-normal mass distribution $y'(D_{ae}) = 1/N_f \times y(D_{ae})$ as a function of the aerodynamic diameter (D_{ae}) using by equation:

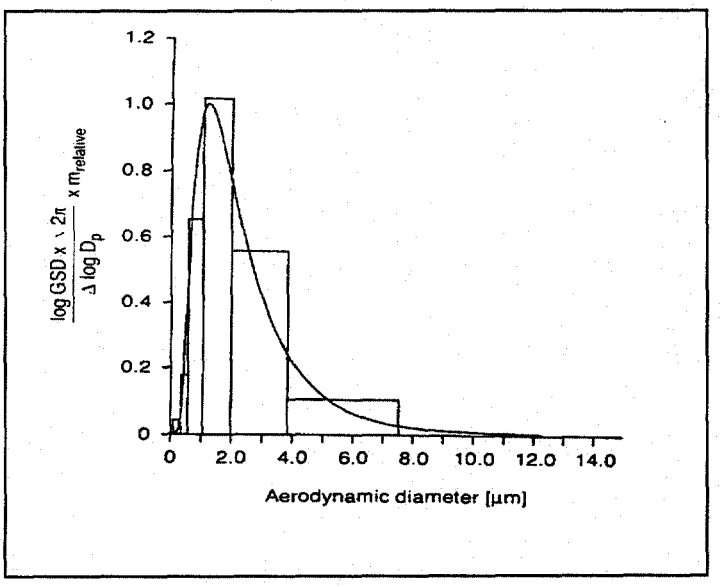
$$y'(D_{ae}) = \exp\left[-\frac{(\log D_{ae} - \log MMAD)^2}{2 \times \log^2 GSD}\right] \qquad (2)$$

and use the normalization factor (N_f):

$$N_f = \left(\frac{\Sigma mass}{\log GSD \times \sqrt{2\pi}}\right)^{-1} \qquad (3)$$

It should be noted that for the graphical display of data the size distributions shown in Fig. 3 is constructed utilizing equation 2.

Figure 5: Principle of characterization of aerosol atmosphere



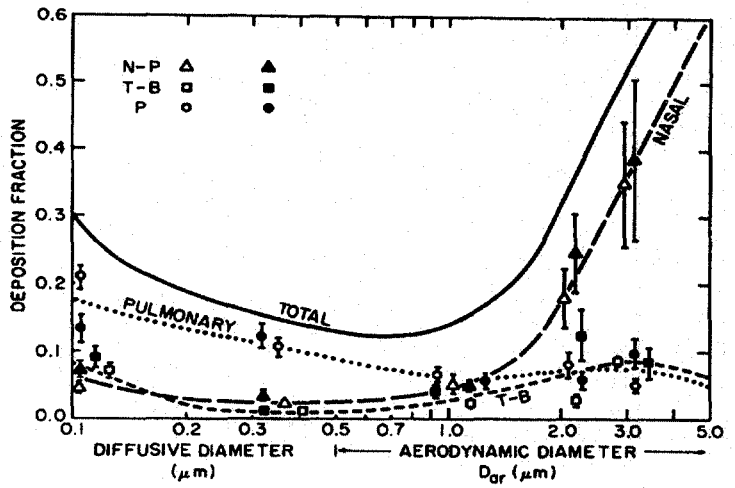
The relative mass with an aerodynamic diameter ≤ 3 µm ("respirable mass fraction") [Raabe, 1982; Snipes, 1989; SOT-Commentary, 1992] is calculated from the regression line. For probit transformation and linear regression FORTRAN algorithms published by Rosiello *et al.* (1977) are used. The MMAD was calculated using published following formulas (Marple and Rubow, 1980; Pauluhn, 1994; USP XXII, 1992).

The algorithm for the calculation of particle size characteristics is taken from pertinent reference works on aerosol physics (Dennis, 1976; Marple and Rubow, 1980) and proves to be generally applicable (Pauluhn 1988; Pauluhn, 1994).

Respirability

Fig. 6 below, demonstrates that the particle-size distribution achieved is adequate to reach all potential target structures of the respiratory tract.

Figure 6: Respirability of Aerosols (Raabe, 1982)



Legend: N-P: Nasopharyngeal, T-B: tracheobronchial, P: pulmonary

6.9. Collection Efficiency

The sampling equipment was adjusted with calibrated flow-meters to internationally recognized standards (ACGIH, 1978; Section I "Calibration of Air Sampling Instruments").

The conditions for generating the test atmosphere are optimized to provide maximum aerosol respirability to rats (Raabe, 1982; Snipes, 1989; SOT-Commentary, 1992). The absence of larger particles and high flow rates in the vicinity of the sampling ports make it possible to disregard potential anisokinetic sampling errors, thus ensuring a representative sampling even with different sampling probe orifice diameters and flow rates. The tolerance limits for the radius of the probe orifice are calculated using the following formula [ACGIH, 1978]. Calculations consider both a particle size distribution that encompasses aerodynamic diameters (D_{ae}) of 0.5 to 7.4 µm and sample flows ranging from 8 to 80 ml/sec.

$$5 \times \sqrt[3]{\frac{flow \times \tau}{4 \times \pi}} \leq r_p \leq \frac{1}{5} \times 2\sqrt{\frac{flow}{g \times \tau \times \pi}}$$

r_p = radius of the sample probe in cm = ½ x D_p
τ = relaxation time ($D_{ae\ 0.5\ \mu m} = 1x10^{-6}$ sec; $D_{ae\ 7.4\ \mu m} = 1.7x10^{-4}$ sec)
g = gravity constant = 980 cm/sec^2

Tolerance limits calculations for the sample probe orifice (r_p) indicated that a representative sampling is assured when the orifice inner diameter is in the range of 1.0 to 1.6 cm. Orifices of the sampling instruments used here are in compliance with this criteria. Details of the D_p tolerance limit calculations are published elsewhere (Pauluhn, 1988; Pauluhn, 1994).

6.10. Stability of the Test Atmosphere

The integrity and stability of the aerosol generation and exposure system was measured by using a RAM-1 real-time aerosol photometer (MIE, Bedford, Massachusetts, USA). Samples were taken continuously from the vicinity of the breathing zone.

This chamber monitoring allows for an overall survey of toxicologically relevant technical parameters (inlet and exhaust flows as well as atmosphere homogeneity, temporal stability, and generation performance). Interruptions in exposure (e.g. resulting from obstruction of nozzles or other technical mishaps) are recorded and, if applicable, a commensurate interval is added to the exposure duration for compensation.

6.11. Body weights

The body weights were determined prior to induction, on study days three and seven, and, in most instances, weekly thereafter. Animals were also weighed before necropsy.

6.12. Clinical signs

If applicable, the appearance and behavior of each rat was examined carefully before

and after exposure/administration and at least once daily thereafter (including weekends). As can be seen from the Appendix (Scheduling/Activities) on some days no observations were made due to public holidays. Assessments from restraining tubes were made only if unequivocal signs occurred (e.g. spasms, abnormal movements, severe respiratory signs). Following exposure, observations are made and recorded systematically; individual records are maintained for each animal. Cage-side observations included, but were not limited to, changes in the skin and fur, eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous system, and somatomotor activity and behavior pattern. Particular attention was directed to observation of tremors, convulsions, salivation, diarrhea, lethargy, somnolence and prostration.

6.13. Delayed-onset Lung Function Measurements

Measurements were conducted with spontaneously breathing, conscious, unrestrained and spontaneously breathing rats through a barometric whole-body plethysmography system (Buxco, Troy, NY, USA). Measurements commenced shortly after the MDI-challenge. Briefly, each rat was placed in a chamber, and continuous measurement of the box pressure–time wave was made via a transducer connected to a computer data-acquisition system. Measurements focused on changes in RR (respiratory rate), TV' (pseudo-volume), and Penh (enhanced pause).

6.14. Bronchoalveolar lavage

Shortly after exsanguination, the diaphragm was incised and the lungs were allowed to collapse. The excised lungs of the animals were then lavaged twice with 5 ml saline (kept @ 37 °C) per rat and the 2 washings combined. In the bronchoalveolar lavage fluid (BALF) the following indicators of pulmonary effects were addressed: (1) total protein to quantitate increased permeability of the alveolar-capillary barrier, (2) lactate dehydrogenase (LDH) as an index of cytotoxicity, (3) the total number of cells, and (4) cytodifferentiation with particular focus on eosinophilic and neutrophilic granulocytes. For determination, the cellular content of the lavage fluid was removed by centrifugation at 200 g (10-min @ 4 °C), and the cell pellet was re-suspended in Dulbecco's calcium and magnesium containing phosphate buffered saline (PBS) substituted with bovine serum albumin (BSA). The number of cells in BAL, including their

corpuscular volume, were determined in triplicates on re-suspended cells (Schärfe-System, Casy 1, Reutlingen, Germany).

6.15. Immunological Determinations

Total IgE in serum was determined as detailed in the Appendix (IgE: pp. 125).

6.16. Organ Weight

Following exsanguination (see 6.17 Necropsy), the weights of lungs were recorded.

6.17. Necropsy

Intraperitoneal injection of sodium pentobarbital (Narcoren®) was used for euthanasia. The animals were then examined for gross pathologic changes. All findings deviating from normal were documented. The lungs of the exsanguinated animals were weighed. Complete exsanguination was performed by severing the *aorta abdominalis*. Further details concerning the histopathological evaluation are provided in the respective Appendix.

6.18. Histopathology

For the histological examination, the following organ tissues were fixed (details *cf.* 'Histopathological Report' pp. 141). Lungs were examined in all groups after the first and last challenge.

Further details regarding the histological technique and additional stains are provided in the histopathology report.

6.19. Statistical Evaluation

Relative and absolute organ weights, and lavage data were analyzed by a one-way analysis of variance and Tukey-Kramer *post hoc* test (BCTIC Computer Code Collection - Biomedical Computing Technology Information Center: ANOVA a FORTRAN Program to Perform one-way Classification Analysis of Variance. Vanderbilt Medical Center, Nashville, Tennessee, USA).

One-way analysis of variances (ANOVA): In this parametric method, the data are checked for normal distribution by comparison of the median and mean values. The variances between the groups were tested for homogeneity with Box's test. If the F-test showed that the variation within the group was greater than that between the groups, this fact is indicated in the appendix by the remark "no statistical difference between the groups". If a difference was determined, a pairwise *post-hoc* (one and two-tailed) comparison of the groups was performed using the Games and Howell modification of the *Tukey-Kramer* significance test.

Randomization: The randomization lists were produced with the aid of a computer program which used a random number generator.

6.20. Reproduction of Raw Data

Raw data entered into, processed by and/or stored in a computer system could be saved and printed out in various formats. The precision (number of decimal places) of the values printed and reproduced in this report reflect toxicologically relevant levels of precision. Deviations between manually calculated and computer-determined values can arise due to rounding. Values with no decimal places do not necessarily represent the pertinent measurement precision of the detection system.

6.21. Software Programming and Validation

Software code for the following purposes was written in Digital Fortran: particle-size analysis, ANOVA, Fisher test, meta-analysis of pulmonary function data. The computer programs were carefully validated. The validation was conducted using text book data sets (Gad and Weil, 1982). However, it should be taken into account that the formal requirements of the GLP-principles for validation of computer software are not fulfilled. Wherever possible, raw data and calculated values are displayed graphically to provide a versatile opportunity for data comparison.

6.22. Raw Data and Report Archival

The study protocol, raw data, and the final report are retained in the archives specified by Bayer HealthCare, Bayer AG. The storage of a retention sample of the test item and, if applicable, also of the reference item is in the responsibility of the sponsor.

7. RESULTS

7.1. Topical and Inhalation Induction

Four groups of thirtytwo rats each were used in this study. Animals of the control groups were not sensitized (normal housing; control-1 abbreviated C-/-) and were either not challenged or challenged in the same manner as the animals of groups 3 and 4 (normal housing; control-2; abbreviated C-/+); topical administrations were made as follows - day 0: 40 or 150 µl MDI (undiluted) on the dorsal area of the trunk (treated area and dose see Method Section), day 7: booster administration to the contralateral skin of the flank using the same regimen. The 40 and 150 µl MDI treatment groups are abbreviated as topical-low and topical-high. Starting with day 16, rats of all groups (except control-1) were challenged by inhalation to ≈40 mg MDI/m³ for a duration of 30-min on the target days 16, 35, 49, and 69 (the exact challenge schedule is shown on page 44 in the Appendix.

Table 2: Induction of animals

Group	1	2	3	4
	Control-1	Control-2	Topical-low	Topical-high
Topical application:				
Volume of neat MDI per rat (days 0 and 7):	--	--	1 x 40µl 1 x 40µl	1 x 150µl 1 x 150µl

Observations-Induction:

No clinical signs were observed following the induction to MDI. The incidence, intensity, and time course of MDI-related skin lesions are detailed in the Appendix (pp. 56).

Group 3: Application site: partial reddened, application site: partial red encrustations.

Group 4: Application site: partial reddened, application site: partial red encrustations, application site: edema.

Briefly, local responses at the site of induction were observed in group 3 after the booster induction (day 7), whilst in group 4 similar responses occurred after the first induction.

7.2. Inhalation Challenge to MDI-Aerosol

The results of the characterization of atmosphere during the 30-min challenge periods with the MDI-aerosol (hapten) are summarized in Table 3. The target concentration for each challenge was 40 mg MDI/m³, the average challenge concentrations was 37.8±1.08 mg MDI/m³ by filter analysis and 37.4±1.09 mg MDI/m³ by cascade imactor analysis.

Table 3: Generation and characterization of chamber aerosol atmosphere (Challenge)

Group	2-4	2-4	2-4	2	3 & 4
	Challenge				
Week	2	5	7	9	10
Animal nos.	9 - 32	9 - 32	9 - 32	9 - 16	17 - 32
Target concentration (mg/m³)	40	40	40	40	40
Pump-Rate (µl/min)	10	10	10	10	10
Nominal concentration (mg/m³)	149.2	149.2	149.2	149.2	149.2
Gravimetric Conc. (mg/m³)	37.5	39.0	39.0	37.1	36.7
Inlet Air flow (l/min):	15	15	15	15	15
Exhaust Air flow (l/min)	26	26	26	26	26
Temperature (°C)	21.0	21.5	21.6	21.8	22.7
Rel. humidity (%)	15.2	16.6	16.6	8.4	8.4
MMAD (µm)	1.64	1.76	1.55	1.72	1.54
GSD	1.77	1.66	1.73	1.68	1.69
Aerosol Mass < 3 µm (%)	85.5	85.5	88.5	85.9	89.7
Mass recovered (mg/m³)	37.0	38.9	36.0	37.1	37.8

a) Based on filter analyses, -- = not applicable; MMAD = Mass Median Aerodynamic Diameter; GSD = Geometric Standard Deviation

Concentration and particle-size measurements made during or close to the challenge periods were reproducible throughout this study. Accordingly, this data demonstrate that all challenges were made under essentially identical conditions.

For specific information concerning calculations of aerosol MMAD, GSD, and mass dependent size fraction below 3 µm, see Appendix (pp.46).

Characterization of the test atmospheres: Analytical (gravimetric filter analyses) and real-time monitoring of the aerosol test atmospheres from the breathing zone indicated that the exposure conditions were temporally stable over the exposure

period. The concentrations obtained by gravimetric analysis (filter and cascade impactor analyses) demonstrate that all determinations provided virtually identical results.

Temperature values in the inhalation chamber were in the range suggested by the testing guidelines. Humidity values were lower; this is undoubtedly related to the use of dry conditioned air for aerosol dispersion.

7.3. Toxicological Results – Repeated Elicitation

The results obtained during and following the repeated challenge exposures of rats are summarized in Table 4.

Table 4: Summary of morbidity and mortality - Challenge

Group/ Sex	Group	Toxicological Result	Signs on Days	Mortality Day
1 / m	Control-1 (C-/-)	0 / 0 / 8	--	--
2 / m	Control-2 (C-/+)	0 / 3 / 8	37, 63	--
3 / m	Topical-low	0 / 7 / 8	37, 50-51, 70	--
4 / m	Topical-high	0 / 8 / 8	17-18, 36-38, 49, 69	--

m = males, - not applicable, the first study day is day 0

Values given in the 'Toxicological results' column are:
1st = number of dead animals.
2nd = number of animals with signs after cessation of exposure.
3rd = number of animals exposed.

Mortality:

Mortality did not occur in this study.

Signs and observations:

Details concerning signs and observations are provided in the Appendix (pp. 56) in the form of various incidence tables. The following list of signs were observed:

Group 1: No signs were observed following the challenge with MDI.

Group 2: Nasal discharge (serous).

Group 3: Labored breathing patterns, bradypnea, nasal discharge (serous), stridor.

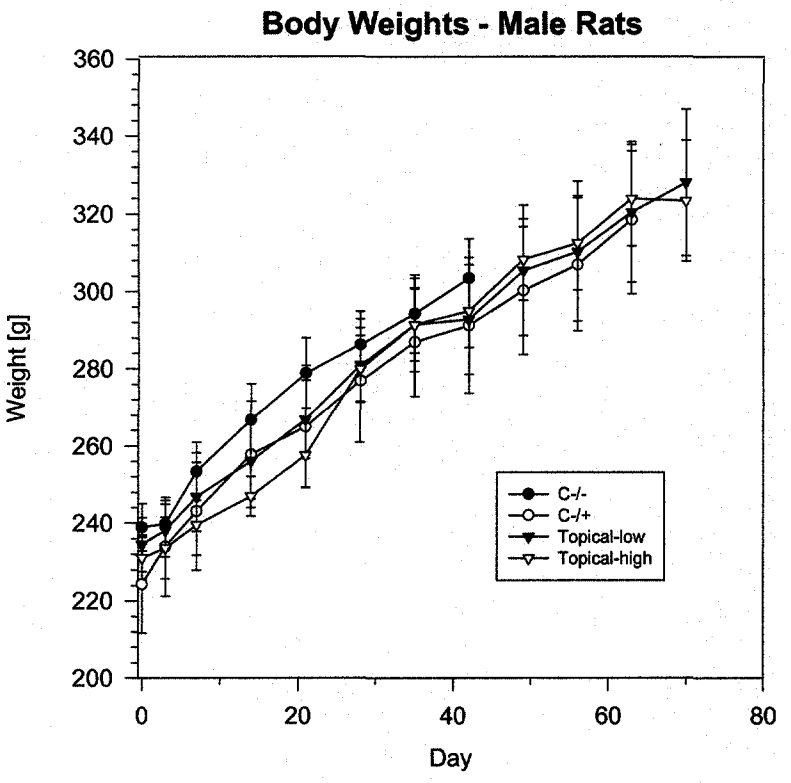
Group 4: Labored breathing patterns, bradypnea, irregular breathing patterns, nasal discharge (serous), tachypnea, stridor, breathing sounds.

Body weights

Individual data and the mean values (± SD) of the body weights are included in the Appendix (pp.84). Mean values (± SD) are summarized in Fig. 7.

Some changes in body weights were observed during the induction period (topical-high only). During the repeated challenge period the body weights of the groups were essentially indistinguishable.

Figure 7: Body weights (means±SD)



7.4. Elicitation of Respiratory Hypersensitivity – MDI-Challenge

All animals of groups 2-4 were simultaneously challenged to the same atmosphere of MDI. Due to technical reasons, measurements of delayed-onset responses were performed before and after challenge in group 4 (all challenge exposures), whilst in group 2 measurements were made before and after the last challenge. Measurements were made in 4 animals per group. In the remaining groups no measurements for delayed-onset reactions were pursued. During or following challenge to approximately 40 mg MDI/m³, the rats sensitized topically and challenged by repeated inhalation exposures displayed a consistent delayed-onset respiratory response which aggravated with increased number of challenges. Data shown in Figs. 8-9 illustrate that with respect to delayed-onset respiratory responses, the rats of group 4 (topical-high) displayed a high incidence of marked respiratory responses.

Figure 8: Area under the curve (AUC) based on changes of Penh (enhanced pause) before and after challenge. The Brown Norway rats of the high dose group were sensitized by topical administration (MDI_{top}: 150 µl on the shaved flanks on days 0 and 7 using three 2 cm spots, each dosed with 50 µl undiluted MDI) and were challenged repeatedly with MDI-aerosol at 39 mg/m³ for 30 min. The rats of the control (C-/+) were not sensitized but were repeatedly challenged similarly to the sensitized animals. Respiratory effects were monitored as described in the legend of Fig. 9. AUCs above the dashed line are considered positive.

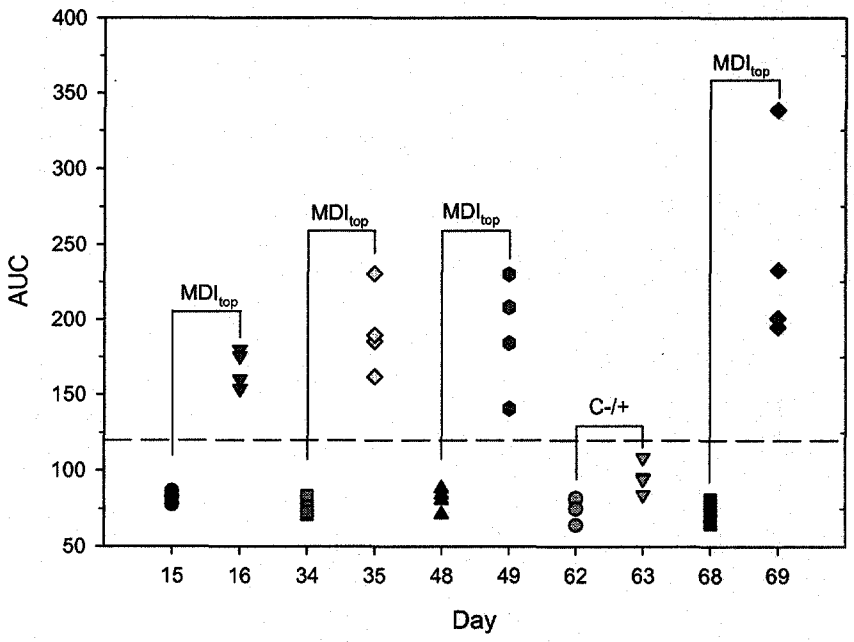
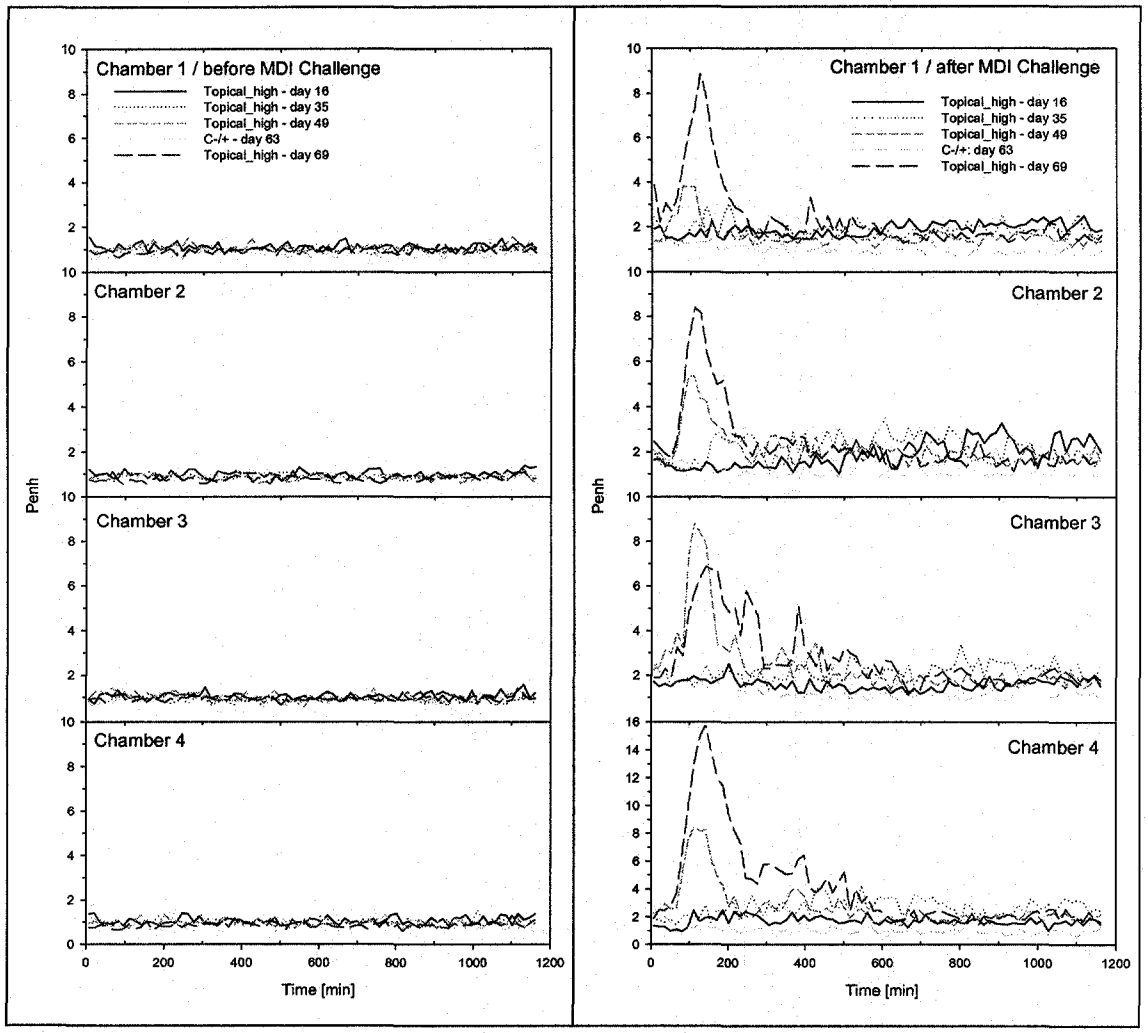


Figure 9: Change of Penh (enhanced pause) in individual Brown Norway rats sensitized by topical administration (150 µl on the shaved flanks on days 0 and 7 using three 2 cm spots, each dosed with 50 µl undiluted MDI) and challenged repeatedly with MDI-aerosol (39 mg/m³) for 30 min. Pulmonary function was monitored in four rats using barometric plethysmographs during a data collection period of approximately 20 hours. Measurements were made 1 day before challenge (left panel) or after each challenge (right panel) in the topical-high group. In the C-/+ group measurements were made only after the day 63 challenge.



7.5. Bronchoalveolar Lavage

The detailed results are summarized in the Appendix (pp. 88). The most salient results are detailed in Table 5 and Figs. 10a,b.

Recovery of bronchoalveolar lavage fluid (BALF) was approximately 80-90% of the instilled volume and was similar amongst the groups. The results summarized in Table 5 show in the topical groups statistically significant changes of most parameters analyzed.

Table 5: Bronchoalveolar Lavage

Parameter	C-/-	C-/+	Topical	
			Low	high
B.W.	306.50	309.00	326.25	322.63
LW-abs	1451.75	1496.86	2174.63**	2156.13**
LW-rel	474.91	484.95	668.96**	667.82**
Recov.	8.75	8.36	8.56	8.38
TCC	6.61	8.57	18.70**	19.20**
MCD	11.83	11.80	11.37**	11.55
MCV	1.00	0.99	0.95	1.04
LDH	71.61	102.03	147.31*	177.51**
PROT	0.38	0.44	0.75**	0.81**
AM	91.13	90.95	76.17**	74.67**
PMN	1.33	2.29	9.29**	9.96**
LYM	0.46	0.48	5.29**	7.92**
EOS	0.79	0.76	3.17**	1.79*
Foamy	4.67	3.71	4.88	4.04
NC	5.57	8.11	10.98	15.63

Abbreviations:
B.W. = Body Weight (bw) - g
LW-abs = Lung weight (absolute) - mg
LW-rel = Lung weight (relative) - mg/100 g bw
Recov. = Recovery of lavage fluid - ml
TCC = Total cell count in BAL - # 10^6/lung
MCD = Mean cellular diameter - um
MCV = Mean cellular volume - 10^-12 L
LDH = Lactate dehydrogenase - U/L
PROT = Protein - g/L
Count = Number of cells counted per cytospot
AM = Alveolar macrophages - %
PMN = Polymorphonuclear cells - %
LYM = Lymphocytes - %
EOS = Eosinophils - %
Foamy = Foamy - %
NC = Cells not classifiable - %
*,** = P < 0.05, P < 0.01
BAL-volume: 10.0 (ml)

Figure 10a: Summary of BALF-parameters

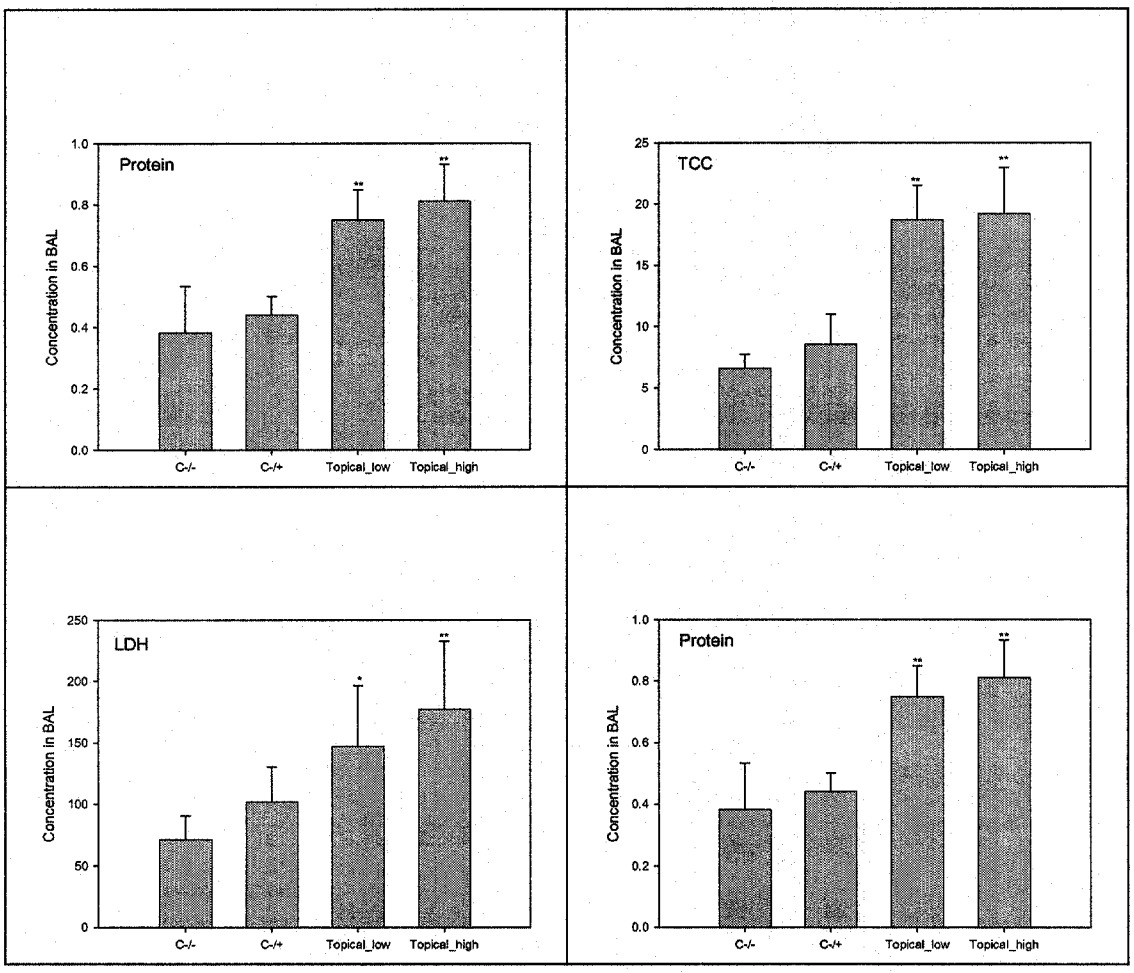
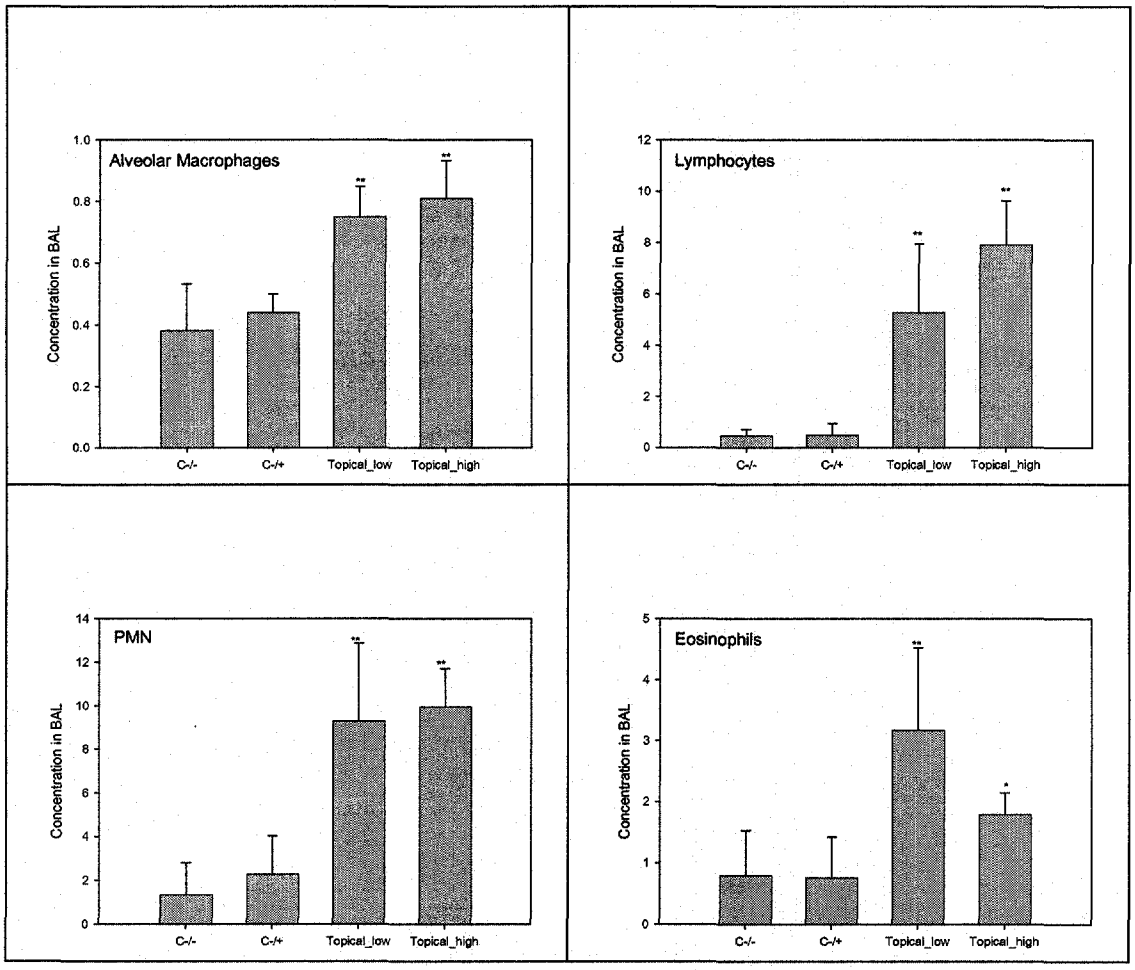


Figure 10b: Summary of BALF-parameters - continuation

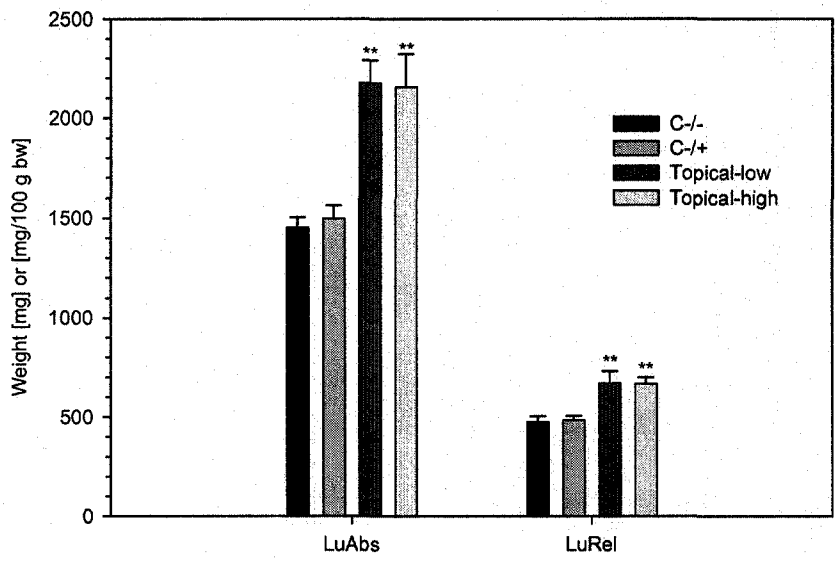


7.6. Lung Weights

The detailed results are summarized in the Appendix (pp. 121) and in Fig. 11.

The body weights at sacrifice did not show any appreciable differences amongst the groups. Absolute and relative lung weights of the topical groups were statistically significant increased.

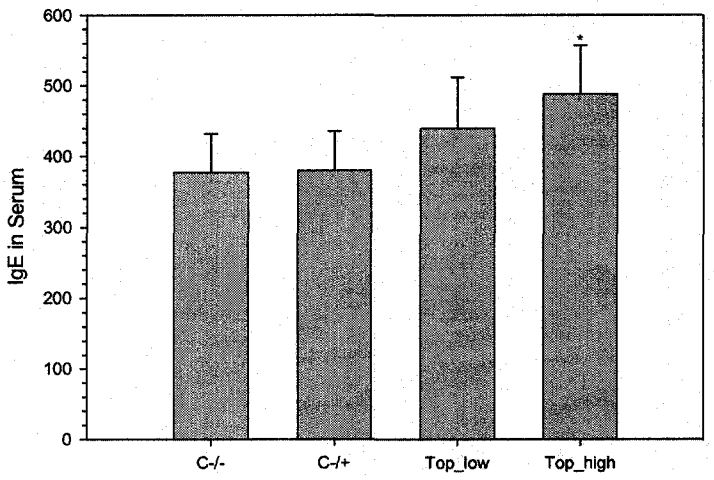
Figure 11: Lung weights at sacrifice (relative and absolute)



7.7. IgE-Determinations in Serum and BALF

The detailed results, including the respective methodological descriptions are summarized in the Appendix (pp. 125). The analysis of data revealed a slight difference amongst the groups which gained statistical significance in the topical-high group.

Figure 12: IgE in Serum



7.8. Necropsy

During necropsy, macroscopical lung findings, e.g. area/s, were seen in the majority of animals from groups 1 and 2 rats and in all from groups 3 and 4. Furthermore, enlarged lung associated lymph nodes from control groups and in all from groups 3 and 4. A list of the individual findings is included in the Appendix.

7.9. Histopathology

The detailed results, including the respective methodological descriptions are summarized in the Appendix (pp.141). The most salient findings are presented in Table 6.

Histopathologically, the lungs were evaluated after H&E and after HEA staining.

Inflammatory findings (focal inflammatory infiltrates, inflammation) were seen in controls as well as in substance-treated rats. However, in substance-treated rats these findings were much more pronounced. These lesions included inflammation of the airways, partly with beginning peribronchial/peribronchiolar fibrosis, alveolar septal thickening, airway epithelial thickening and an increased number of alveolar macrophages, including BALT (bronchus associated lymphatic tissue) activation.

The incidence as well as the grading of vascular hypertrophy was clearly increased in groups 3 and 4. The occurrence of (peri)-bronchial/bronchiolar and perivascular eosinophils was increased in rats from groups 3 and 4. Together with the increased inflammatory reaction, the inflammation related eosinophils were increased in groups 3 and 4. Comparing the lesions from groups 3 and 4, there is no clear difference between these two groups.

Table 6: Summary of significant histopathological findings in the respiratory tract (for a more detailed presentation of data *cf.* to histopathology report)

		Day 66			
Finding	**Group**	**C-/-**	**C-/+**	**Top_low**	**Top_high**
LUNG – Day 65		(8)	(7)	(8)	(8)
Bronchial epithelial thickening					
	Grade 2	0	0	7	3
	Grade 3	0	0	1	5
(Peri-)Bronchial eosinophils					
	Grade 2	1	1	6	8
	Grade 3	0	0	2	0
BALT activation					
	Grade 2	0	2	3	4
	Grade 3	0	0	4	4
Inflammation					
	Grade 1	1	0	3	7
	Grade 2	0	0	5	0
(Peri-)Vascular eosinophils					
	Grade 1	5	5	1	0
	Grade 2	2	1	7	8

8. DISCUSSION

The results of study can be summarized as follows: During the sensitization phase some animals displayed dose-dependent local effects at the site of induction. After challenge transient breathing responses were observed. In topically sensitized rats, a time-related exacerbation of delayed-onset responses was observed. Pulmonary inflammation was indicated by most endpoints determined in bronchoalveolar lavage. This included elevated protein, increased numbers of neutrophilic and eosinophilic granulocytes and lymphocytes. Histological inflammatory findings (focal inflammatory infiltrates, inflammation) were seen especially in sensitized rats. These lesions included inflammation of the airways, partly with beginning peribronchial/peri-bronchiolar fibrosis and included alveolar septal thickening and BALT activation.

IgE determinations revealed slightly elevated levels in the topical sensitization groups. In the topical-high group the increase gained statistical significance.

In summary, the findings of this study support the conclusion that the Brown Norway rat model is suitable to identify MDI as an agent causing a pulmonary inflammatory response upon topical induction followed by repeated inhalation challenge exposures. Consistent and unequivocally positive delayed-type changes of breathing patterns were observed. These findings suggest that MDI promotes a more delayed-onset type rather than immediate-type inflammatory response.

9. KEY TO ABBREVIATIONS IN TABLES

PIF	Peak inspiratory flow
PEF	Peak expiratory flow
MV	Minute volume
TV	Tidal volume
IT	Inspiration time
ET	Expiration time
RR	Respiratory rate
ECD	Effective cut-off diameter
STAND, S, Std, SD	Standard deviation (σ)
MW/MEANS, x	Means
B.W.	Body weight
F	F test value (F ratio)
DF	Degrees of freedom
PROB	Probability
SS	Total sum of squares
MS	Mean squares
TREATMENT	- Between the groups
ERROR	- Within the groups
TOTAL	- Total

Lung weights

Absolute weights in milligrams
Relative weights in milligrams per 100 grams of body weight

10. REFERENCES

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ASTM - Standard Test Method for Estimating Sensory Irritancy of Airborne Chemicals. ASTM Designation: E 981-84. American Society for Testing and Materials, Philadelphia, USA.

BCTIC Computer Code Collection - Biomedical computing Technology Information Center, ANOVA a Fortran Program to Perform one-way Classification Analysis of Variance. Vanderbilt Medical Center, Nashville Tennessee, U.S.A.

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